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The rare sugar D-allose has a reducing effect against ischemia-reperfusion injury on the rat abdominal skin island flap model

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ARTICLE INFO

Article history:
Received 21 December 2012
Received in revised form
20 February 2013
Accepted 1 March 2013
Available online 21 March 2013

Keywords:
D-allose
Rare sugar
Ischemia-reperfusion injury
Skin island flap
Antioxidant
Myeloperoxidase (MPO)
Neutrophil

ABSTRACT

Background: Recently, one of the rare sugars, D-allose, has received attention from many researchers because of its availability for mass production and its various physiological functions. Among these, an antioxidative effect has been strongly suggested. In this study, we investigated whether this effect is also applicable to the field of skin surgery.

Methods: In ischemia-reperfusion injury model using the rat abdominal skin island flap (male Wistar rats, $n = 110$), D-allose was injected intravenously 15 min before 8-h ischemia. The survival area (%) was measured by digital photographic assessment 1 wk after surgery, and multiple comparisons (Fisher's protected least significant difference) were carried out. Histopathological examination (neutrophilic infiltration into dermis in hematoxylin and eosin stain) and immunostaining (of ectodermal dysplasia-1 (ED1)-positive cells/flap) were assessed. Myeloperoxidase (MPO) activity in the skin flap (sampling at the time of 8 h after reperfusion) was measured spectrophotometrically, and Student *t*-test was performed.

Results: D-allose extended the survival of the remaining flaps, and a dose greater than 30 mg (0.1 mg/g) was necessary to be effective. The flap survival rates in the 30, 60, and 150 mg groups were significantly higher than that in the control (saline) group: 75.87 ± 5.90, 79.27 ± 7.81, and 77.87 ± 6.20 versus 50.53 ± 9.66, respectively ($P < 0.05$). ED1-positive cells/flap in 60 mg of D-allose and control (saline) were 78 ± 25.7 *versus* 124 ± 15.8, respectively ($P = 0.08$). The MPO activity in the D-allose 60 mg group was 0.40 ± 0.04, and that in the control (saline) was 0.72 ± 0.12. D-allose significantly reduced the skin tissue MPO activity ($P < 0.05$) compared with that in the control (saline) group.

Conclusions: We proved that D-allose has a reducing effect against ischemia-reperfusion injury on the skin island flap model, and the mechanism is related to inhibiting the activity of neutrophils in the skin tissues. Compared with chemo-synthetic materials,

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0022-4804/$ – see front matter
http://dx.doi.org/10.1016/j.jss.2013.03.006

rare sugars are safer for our bodies as well as the environment; therefore, this rare sugar project is expected to lead to the development of a safer antioxidant for skin flap surgery.

1. Introduction

Rare sugars are defined as monosaccharides and their derivatives that exist in nature in very small quantities. Although many kinds of rare sugars are known (more than 50), almost none of these are commercially available. Even if some of these sugars were available, they would be too expensive, and obtaining a sufficient supply for basic research would be difficult. After accumulating evidence from continuous rare sugar research over many years in the Faculty of Agriculture, Kagawa University, the structural relationship among all monosaccharides was systematized as Izumoring [1] (Fig. 1) by the discovery of D-tagatose-3-epimerase [2], which is a new enzyme inducing rare sugars. Through this discovery, the mass production technology of rare sugars has been established [3], and the field of basic research on the physiological activities of rare sugars has become widespread. As shown in Figure 2, D-psicose is produced from D-fructose by the enzymatic reaction of D-tagatose-3-epimerase, and then D-allose is synthesized in a large amount from D-psicose using L-rhamnose isomerase [4]. D-allose is the C3-epimer of D-glucose (Fig. 2). Among its various activities, it has become clear that D-allose has inhibitory effects on cancer cell proliferation [5] and the production of reactive oxygen species [6] as well as a protective effect against ischemia-reperfusion injury [7–9].

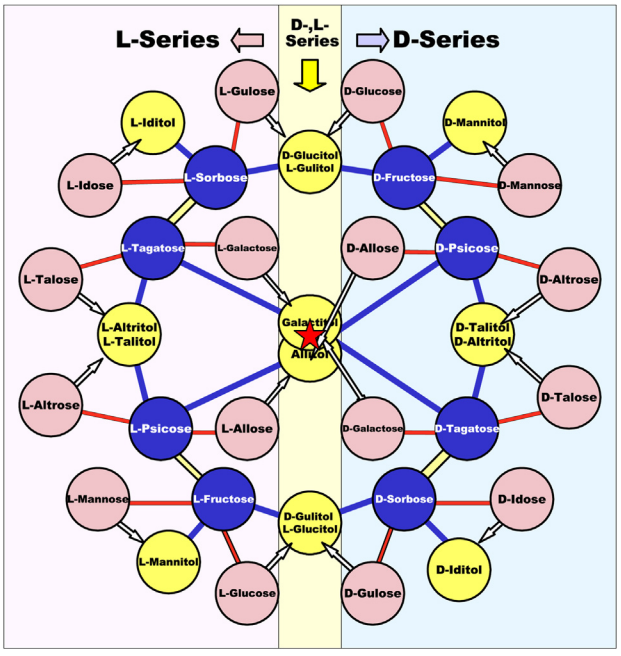

Fig. 1 – Izumoring: Structural relationship among all monosaccharides, which was systematized by Izumori [1]. The location of rare sugars such as D-allose is easily visualized. (Color version of figure is available online.)

Numerous experimental studies have been reported on the mechanism of ischemia-reperfusion injury of skin island flaps and the preventive therapies against flap necrosis using antioxidants [8–15]. However, these antioxidants have not been applied clinically yet [14] because of excess toxicity or limited efficacy. On the basis of this background, we investigated whether the antioxidative effect of rare sugar D-allose is also applicable to the field of skin surgery. If its application can be developed, rare sugar D-allose ($C_6H_{12}O_6$) will be useful and safer for the ecosystem than other existing antioxidants [2,8]. Therefore, in this study, we examined the reducing effect of D-allose against ischemia-reperfusion injury on a rat abdominal skin island flap model, and investigated the mechanism of the antioxidative effect.

2. Materials and methods

2.1. Experimental design

Rare sugar D-allose was supplied by Kagawa University Rare Sugar Research Center, Kagawa, Japan. Other reagents were purchased from Kagawa Science Co Ltd, Takamatsu, Japan. Male Wistar rats (n = 110, 280–300 g) were purchased from WKY, Charles River Japan Inc (Osaka, Japan). Rats were handled in compliance with the Guide for Experimental Animal Research and the experiment was approved by the Institutional Animal Care and Use Committee of the Kagawa University. Ninety rats were used in Experiment 1, 10 rats each were used in Experiments 2 and 3. In Experiment 1, the 90 rats were divided into six groups (n = 15, each group) as follows: (1) D-allose 150 mg (0.5 mg/g), (2) D-allose 60 mg (0.2 mg/g), (3) D-allose 30 mg (0.1 mg/g), (4) D-allose 15 mg (0.05 mg/g), (5) D-glucose 60 mg (0.2 mg/g), and (6) saline: control. In Experiments 2 and 3, the 10 rats were divided into two groups (n = 5, each group) as follows: (1) D-allose 60 mg (0.2 mg/g) and (2) saline: control. D-allose and D-glucose were dissolved in

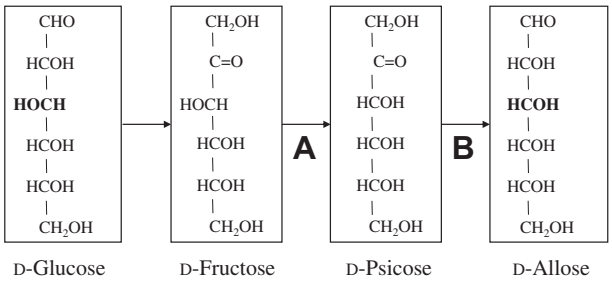

Fig. 2 – Structure of D-allose together with D-glucose, D-fructose, and D-psicose. D-fructose is converted to D-psicose and then D-psicose is converted to D-allose by the enzymatic reactions of D-tagatose 3 epimerase (A) and L-rhamnose isomerase (B), respectively.

distilled water, and the injected amount in all six groups was equal to 0.6 mL.

2.2. Surgical procedure

Pentobarbital (35 mg/kg) was intraperitoneally injected into rats for anesthesia. The hair was removed from the right abdominal/groin area, where an island flap measuring 3 × 5 cm was raised and supplied by the inferior epigastric artery and vein. The femoral artery and vein were ligated and cut distal to the origin of the epigastric artery and vein, which resulted in direct perfusion of the flap only by the femoral vessels (Fig. 3). The raised skin island flap was replaced in the original position and fixed with 4-0 nylon stitch. D-allose was injected into the left femoral vein using a 30G needle. Fifteen minutes after injection, temporary ischemia was induced by occluding the right femoral vessels with a microvascular clip. After 8 h, the microvascular clip was removed, and the ischemia-reperfusion injury model using the rat skin island flap was completed. A laser-Doppler blood-flow meter (FLO-C1FM; OMEGAWAVE Inc, Fuchu, Japan) was used to confirm both ischemia and reperfusion conditions.

2.3. Measurements

2.3.1. Experiment 1: Survival of remaining flaps

After 1 wk, viability of the flap was observed and judged. The flap was photographed using a digital camera, and the survival area (%) was measured using area-calculating software on a computer and expressed as a percentage of the total flap. The mean percentage of viability was calculated for each experimental group.

2.3.2. Experiment 2: Histopathological examination and ectodermal dysplasia-1 (ED1) immunostaining

Skin specimens were taken from the most distal end of the flaps 8 h after reperfusion, and were placed in 10% formalin and stained with hematoxylin-eosin stain to evaluate flap histopathology. Four-micrometer thick sections were evaluated at ×400 magnification and ED1-positive cells were assessed using primary antibodies (mouse anti-rat; 1:500); no overlapping fields were recorded.

2.3.3. Experiment 3: Myeloperoxidase activity

At the time of 8 h after reperfusion, skin tissue measuring 1.5 × 1.5 cm was extracted from the center of the skin island flap. The skin tissue was weighed and homogenized with hexadecyltrimethylammonium bromide buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0) with a Polytron homogenizer (Brinkmann, Westbury, NY), and myeloperoxidase (MPO) activity was measured spectophotometrically by the method of Krawisz *et al.* [16].

2.4. Statistics

Data are presented as means ± SE. Mean percent (%) survival of the skin island flap and standard error were analyzed by one-factor ANOVA. Comparisons among the groups were analyzed by multiple comparison (Fisher's protected least significant difference) analysis. Student *t*-test was used for the intergroup comparisons of MPO activity test. When the *P* value was less than 0.05, the difference was regarded as significant.

3. Results

3.1. Survival of remaining flaps

Mean percent (%) survival of skin island flap is shown in Figure 4, and the data analysis is shown in the Table. As shown in the Table, groups 1, 2, and 3, which were given D-allose at doses of 30 mg (0.1 mg/g) or more, showed significant differences from group 6 (control), which was given saline: 77.87 ± 6.20, 79.27 ± 7.81, and 75.87 ± 5.90 *versus* 50.53 ± 9.66, respectively ($P < 0.05$). In addition, groups 1 and 2 were significantly different from group 5, which was given 60 mg of D-glucose: 77.87 ± 6.20 and 79.27 ± 7.81 *versus* 53.73 ± 10.57, respectively ($P < 0.05$). However, group 4 (62.47 ± 8.36), which

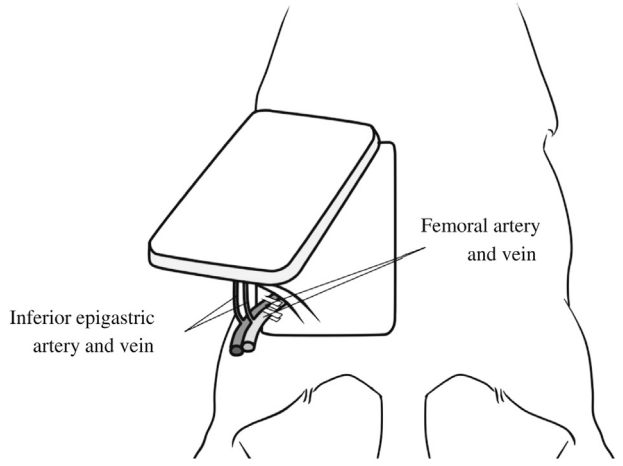

Fig. 3 – Abdominal skin island flap model in which a flap measuring 3 × 5 cm is elevated on the right abdominal/groin area, and is directly supplied only by the femoral vessels.

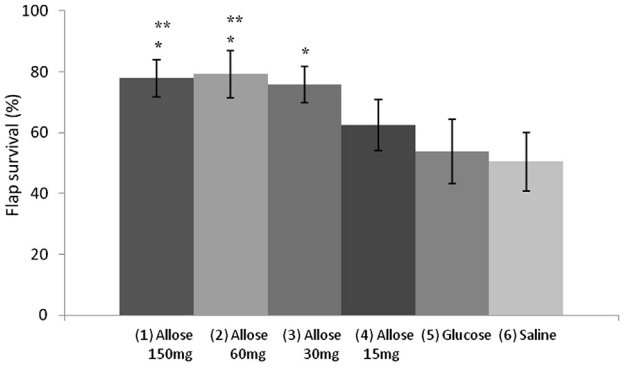

Fig. 4 – Results of Experiment 1: percent (%) survival of the skin island flap. The data are expressed as means ± SEM. *P < 0.05 *versus* saline (control), ** P < 0.05 *versus* glucose.

Table – Mean percent (%) survival of skin island flap and standard error were analyzed by one-factor analysis of variance (ANOVA). Comparisons among the groups were analyzed by multiple comparison (Fisher's protected least significant difference) analysis.

Groups	*n*	% Flap survival (mean ± SE)	Significant difference between groups	Significance (*P* values)	
(1) D-allose 150 mg (0.5 mg/g)	15	77.87 ± 6.20	(1) Allose (150 mg) vs (2) Allose (60 mg)	-	
(2) D-allose 60 mg (0.2 mg/g)	15	79.27 ± 7.81	(1) Allose (150 mg) vs (3) Allose (30 mg)	-	
(3) D-allose 30 mg (0.1 mg/g)	15	75.87 ± 5.90	(1) Allose (150 mg) vs (4) Allose (15 mg)	-	
(4) D-allose 15 mg (0.05 mg/g)	15	62.47 ± 8.36	(1) Allose (150 mg) vs (5) Glucose	$P < 0.05$	
(5) D-glucose 60 mg (0.2 mg/g)	15	53.73 ± 10.57	(1) Allose (150 mg) vs (6) Saline	$P < 0.05$	
(6) Saline (control)	15	50.53 ± 9.66	(2) Allose (60 mg) vs (3) Allose (30 mg)	-	
			(2) Allose (60 mg) vs (4) Allose (15 mg)	-	
			(2) Allose (60 mg) vs (5) Glucose	$P < 0.05$	
			(2) Allose (60 mg) vs (6) Saline	$P < 0.05$	
			(3) Allose (30 mg) vs (4) Allose (15 mg)	-	
			(3) Allose (30 mg) vs (5) Glucose	±	($P = 0.061$)
			(3) Allose (30 mg) vs (6) Saline	$P < 0.05$	
			(4) Allose (15 mg) vs (5) Glucose	-	
			(4) Allose (15 mg) vs (6) Saline	-	
			(5) Glucose vs (6) Saline	-	

was given D-allose at 15 mg (0.05 mg/g), did not show a significant difference from group 6 (saline: control) or group 5, which was given D-glucose at 60 mg (0.2 mg/g). Therefore, producing different results from D-glucose, D-allose extended the survival of the remaining flaps, and a dose greater than 30 mg (0.1 mg/g) was necessary to be effective. This outcome can be seen clearly in Figure 4.

3.2. *Histopathological examination and ED1 immunostaining*

The histopathological findings are shown in Figure 5. Compared with the control (A), D-allose (B) had a a tendency to inhibit neutrophilic infiltration into dermis. Histopathological analysis revealed reduced ischemia-reperfusion injury with D-allose treatment. The ED1 immunostaining result is shown in Figure 6. ED1-positive cells/flap in D-allose group was 78 ± 25.7, whereas the value in the control (saline) was 124 ± 15.8. The immunostaining suggested that D-allose inhibited inflammatory cell infiltration into the dermis although mean count only showed a tendency to decrease ($P = 0.08$).

3.3. *MPO activity*

The MPO activity (mU/mg) in the tissue is shown in Figure 7. The MPO activity in the D-allose group was 0.40 ± 0.04, and that in the control (saline) was 0.72 ± 0.12. D-allose significantly reduced the skin tissue MPO activity ($P < 0.05$) compared with that in the control (saline) group.

4. Discussion

As a result of several experiments on other organs [5–9], it has been suggested that D-allose has a 'unique' antioxidative effect. The reason for describing this effect as 'unique' is that D-allose not only has the ability to remove oxidants (proving an antioxidative effect in the narrow sense), but also reduces

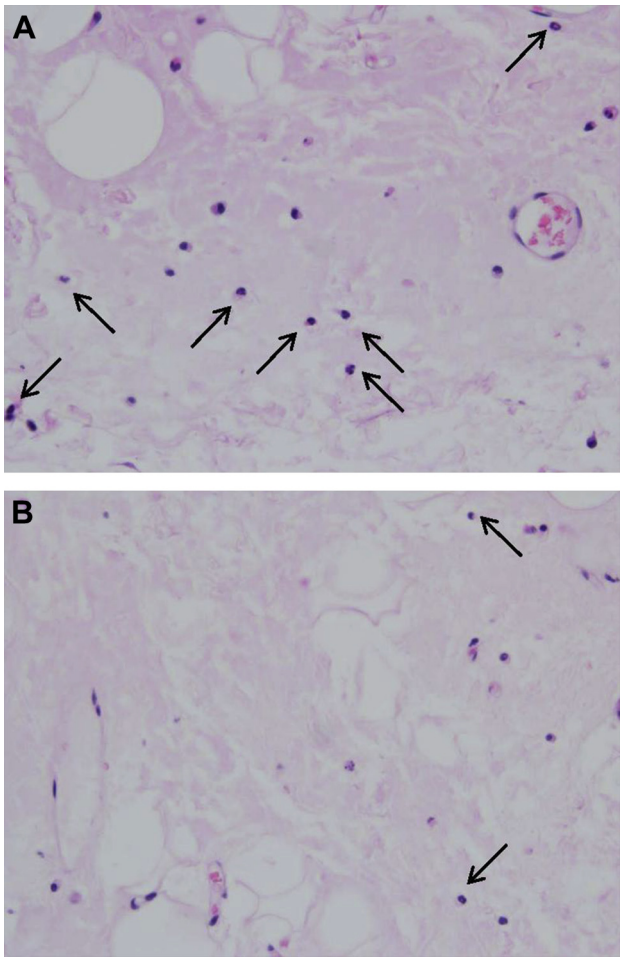

Fig. 5 – Histopathological findings (hematoxylin and eosin stain): skin specimens were taken from the most distal end of the flaps 8 h after reperfusion; (A) control (saline), (B) D-allose (0.2 mg/g). Compared with control (A), D-allose (B) seemed to have a tendency to inhibit neutrophilic infiltration (arrows) into dermis. (Color version of figure is available online.)

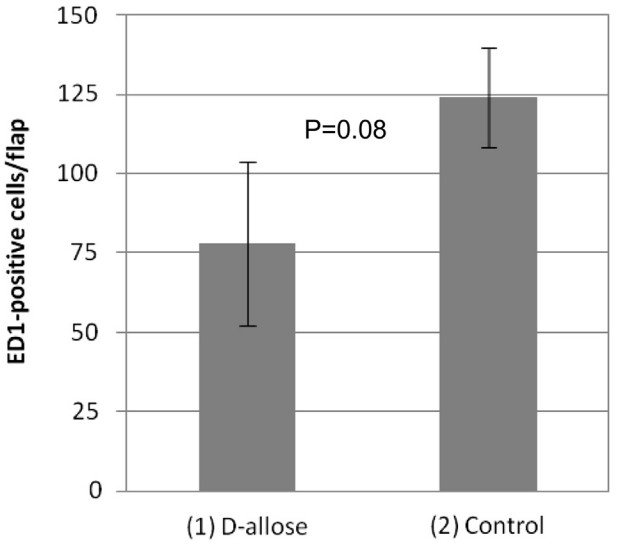

Fig. 6 – Results of Experiment 2: ED1-positive cells/flap. Although only a tendency was shown ($P = 0.08$), D-allose decreased the mean count of ED1-positive cells/flap compared with that in the control group.

the production of oxidants, which is an ability that is rare in other anti-oxidants. This combination of effects is considered to reduce the level of reactive oxygen species, which are present in large quantities during ischemia-reperfusion injury. Expecting this unique antioxidative effect to be stronger than that of other known antioxidants, we applied D-allose to the field of skin surgery.

From the results of Experiment 1, it became clear that D-allose reduced ischemia-reperfusion injury on the skin island flap model. Histopathological examination and ED1 immunostaining (Experiment 2) also suggested that D-allose inhibited neutrophilic infiltration into dermis (Figs. 5 and 6).

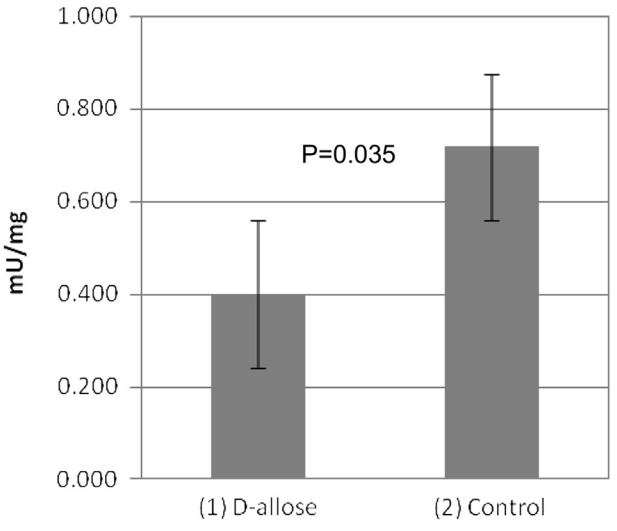

Fig. 7 – Results of Experiment 3: MPO activity in the skin tissues. D-allose significantly reduced the MPO activity ($P < 0.05$) compared with that in the control group.

Subsequently, its mechanism had to be precisely determined by measuring several kinds of antioxidative stress index. We first investigated the level of MPO, which is an enzyme that is mainly released from neutrophils (Experiment 3). Activated neutrophils can secrete several enzymes, such as MPO and elastase, which are directly involved in tissue injury, and the MPO assay is a widely used method to quantify neutrophils as an index of inflammation and oxidative stress in tissues and cells [16]. From the results of Experiment 3, it became clear that D-allose reduced the MPO activity in the skin flap, indicating that D-allose inhibits the activity of neutrophils in the skin tissues.

Once the mechanism of the therapeutic effects of D-allose against skin ischemia-reperfusion injuries becomes clear, the most effective application method should be determined. In this study, D-allose was given intravenously just after elevating the skin island flap (15 min before clipping the vascular pedicle). From the results of Experiment 1, a dose-dependent antioxidative effect for skin ischemia-reperfusion injury was identified, and more than 0.1 mg/g (in this study, 30 mg: group 3) was required. From our previous experiments on other organs, such as liver and brain [8,9], concerning the dose, the same results were obtained, and it has also been proven that a dose more than 4 or 5 times this dose (0.1 mg/g) does not increase the antioxidative effect. Therefore, we set the dose of Experiments 2 and 3 to 0.2 mg/g (60 mg). (Our previous examinations [8,9] were performed at approximately this dose). However, owing to the *in vivo* half-life or duration of physiological activity (about 30 min), another time point (such as just before removing the clip) or route (such as topical administration) may be more effective. Therefore, we are planning further experiments to clarify the appropriate application method of D-allose prior to clinical application.

Recently, a large, rare sugar project has been developed in Kagawa prefecture, Japan. In this project, rare sugars have been produced from monosaccharides such as D-glucose and D-fructose that exist in large quantities in nature, allowing research on the various physiological actions and their mechanism, and finally connecting the benefits to practical applications. Compared with chemosynthetic materials, rare sugars are safer for human beings and gentler for the environment, considered to have few or no side effects [2,8]. In the future, we expect that this area of research will lead to longer preservation of amputated limbs and emergency replantation surgery. In conclusion, we proved that D-allose has a reducing effect against ischemia-reperfusion injury on the rat skin island flap model, and that the mechanism is related to inhibiting the activity of neutrophils in the skin tissues.

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